Chapter 10

Tissue-Print Immunodetection of Transgene Products in Endosperm for High-Throughput Screening of Seeds

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Summary

This method allows high-throughput qualitative screening to identify seeds containing a transgene product in endosperm tissue. It is particularly useful for determining genetic segregation ratios or identifying seeds to be advanced in a breeding program. Tissue printing is used to avoid time-consuming extraction steps. Antibody-based detection of the transgene product makes this method suitable to any transgene product for which a specific antibody is available. It is possible to screen thousands of seeds per week using this method.

Keywords: Seed, Endosperm, Antibody, Protein

1. Introduction

The primary function of seed endosperm tissue is to accumulate reserves for the germinating seedling. This storage function makes endosperm an attractive target for accumulation of transgene products, because foreign proteins that accumulate in a storage tissue are less likely to interfere with development than those accumulating in other tissues. Endosperm-expressed transgenes have been used to improve grain quality *(1–4)* and as a vehicle to produce high-value proteins such as vaccines *(5)* and other high-value proteins *(6–10)*. The seed storage protein promoters are among the strongest in the plant and several have been shown to function well in transgenes.

Transgenes often do not confer a visible phenotype to the plant, but breeding and genetic experiments often require selection of transgenic plants from segregating populations.

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Therefore, laboratory methods for selection of transgenic plants are required. Selection at the seed stage is advantageous because plants that do not express the transgene are identified before planting and therefore do not need to be grown, minimizing the number of plants that need to be grown. Evaluation of seeds is also convenient because seeds can be stored many years and analyzed at a time that is convenient for the researcher.

Immunological detection of the transgene product can be used to identify transgenic seeds if an antibody that reacts with the transgene product is available. Immunological methods are attractive because general protocols *(11)* such as western blotting or ELISA can be applied to different transgene products using different antibodies specific for each transgene product of interest. This minimizes the number of protocols required when evaluating transgenes with a variety of different products.

This chapter describes a high-throughput method for identifying transgenic seeds containing endosperm-expressed transgene products. This method is not a quantitative measure of transgene levels, but it is useful for determining segregation ratios and selecting transgene-positive seeds for planting. While the pericarp is damaged by this method, we plant analyzed seeds without treatment and routinely get >80 germination. This method meets the high-throughput needs of breeding and genetics programs to identify seeds carrying a functional transgene.

2. Materials

1. Sand Paper, 150 grit.
2. Modeling clay.
3. Seed extraction buffer: 65.5 mM Tris–HCL, 3.33% SDS, 5% 2-mercaptoethanol, pH 6.8 (*see* **Note 1**).
4. Nylon-backed nitrocellulose membrane.
5. Blocking solution: 5% (w/v) skim milk powder in 1× PBS (137 mM NaCl, 2.7 mM KCl, 1.4 mM NaH_2PO_4, 4.3 mM Na_2HPO_4, pH 7.4).
6. Transgene product-specific antibody (*see* **Note 2**).
7. Antibody detection reagents: Many systems are available for this. We use an alkaline phosphatase labeled secondary antibody and a colorimetric detection kit (both from Bio-Rad, Hercules, CA) according to the manufacturer's directions.

3. Methods

Screening seeds by sampling the endosperm is desirable because it yields a small amount of tissue for analysis and the seed remains viable for planting. Removing the tissue and extracting the compounds of interest can be time consuming because it involves collecting and weighing tissue, extraction, and centrifugation. Tissue-printing involves first exposing the tissue of interest. We do this by abrading the seed with sand paper. The exposed surface is then moistened with the extraction buffer and pressed onto blotting media. Enough of the transgene product is transferred to the paper to be detected with immunological methods. Because it is not necessary to collect and extract the tissue, tissue-printing is much faster than traditional extraction methods. We routinely process 500 seeds in a batch.

Positive and negative controls should be included on each blot. High background signal and levels of the transgene product near the limit of detection of the method can make distinguishing positive and negative tests difficult. Background signal can sometimes be reduced using the optional protocol for preadsorption of the antibody with nontransgenic corn endosperm.

3.1. Tissue-Print Blotting

1. Prepare the seeds by numbering them with a pencil.
2. Cut the nylon-backed nitrocellulose membrane to the size of the tray you will be processing the blot in. Draw a 1-cm grid on the paper with a soft pencil. Wear gloves when handling the membrane.
3. Make a long (about 2 cm/seed), 5–7-mm diameter cylinder of modeling clay. Grasp a seed firmly between thumb and forefinger with the tip cap oriented toward the hand so that the crown of the seed is exposed. Abrade the seed by rubbing it on a piece of sand paper placed on the laboratory bench so that about 1 mm of tissue is removed to create a flat top on the seed. Use a different place on the sand paper for each seed to avoid contamination between seeds.
4. Press the seed into the modeling clay with the abraded surface up (**Fig. 1**).
5. Add 3–5 ml (depending on seed size) of seed extraction buffer to the sanded surface of the seed.
6. Wait for 2–3 min and add seed extraction buffer as before.
7. Wait approximately 1–2 min and then press the abraded surface of each seed firmly into one of the squares marked on the nitrocellulose membrane (*see* **Note 3**).
8. Allow the membrane to dry. It can be stored indefinitely before processing.

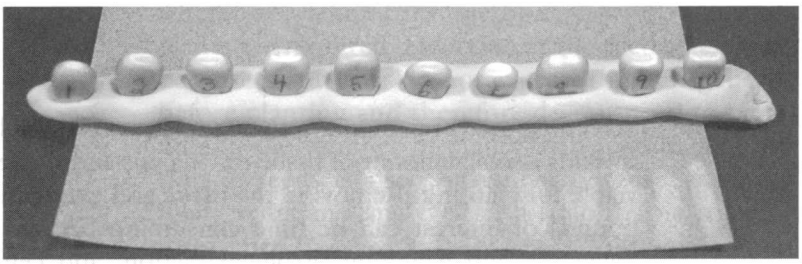

Fig. 1. Abraded kernels in modeling clay ready for addition of seed extraction buffer. The clay is resting on the sandpaper used to abrade the kernels. Marks on the sandpaper are from abrading the kernels.

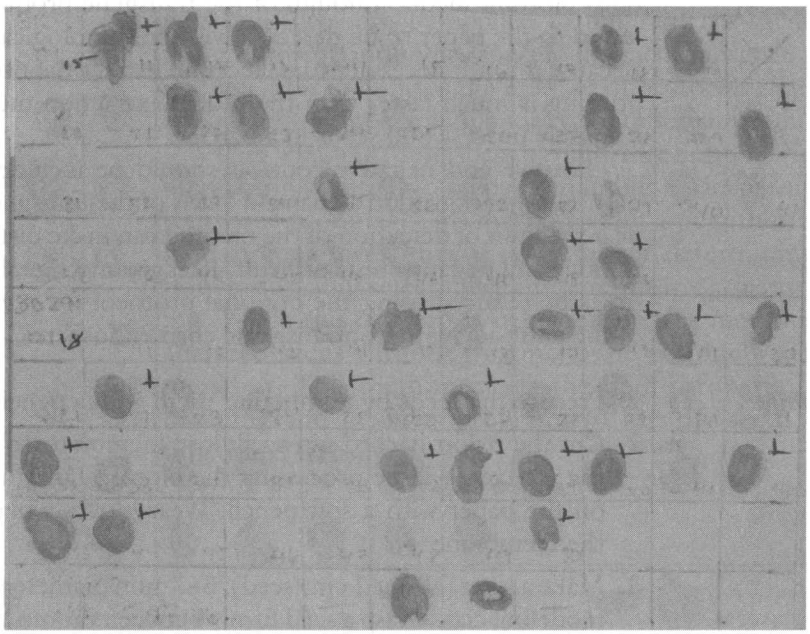

Fig. 2. A typical blot developed with an alkaline phosphatase-conjugated secondary antibody and colorimetric detection system. A plus sign indicates seeds that are considered to contain the transgene product. The bottom row was blotted with two positive control kernels (*bottom center*) and two negative control kernels *(bottom right*).

9. Process the membrane by placing it in a shallow tray containing blocking solution so that the membrane is just covered by the solution. Rock gently for 1 h.
10. Add the antibody to the solution covering the membrane. If using a preadsorbed antibody (*see* **Subheading 3.2**), replace the blocking solution with the preadsorbed antibody. Otherwise, the antibody can be added directly to the blocking solution (*see* **Note 4**). Allow the antibody to bind with gentle rocking for 5 h.
11. Develop the blot using your choice of antibody detection reagents according to the manufacturer's protocol. We use

an alkaline phosphase-labeled goat secondary antibody (Bio-Rad, Hercules, Ca) and a colorimetric alkaline phosphatase detection kit (Bio-Rad, Hercules, Ca). A typical blot is illustrated in **Fig. 2**.

3.2. Preadsorption to Remove Nonspecific Antibodies (Optional)

1. Soak nontransgenic kernels in water for 5 min and manually peel off the pericarp. Remove the embryos using a razor blade or scalpel. Grind the remaining tissue (mostly endosperm) finely in a coffee grinder or mortar and pestle.
2. Extract this ground tissue at 100 mg tissue/ml of seed extraction buffer for 20 min to 1 h. The total volume should be sufficient to just cover the blots that you intend to process with this antibody. Centrifuge and remove supernatant.
3. Add the antibody to this solution and incubate for 5 h. The resulting solution can be added to the blocked membrane.

4. Notes

1. 2-Mercaptoethanol is volatile and toxic. Add it to the solution immediately prior to use. Wear gloves and work in a fume hood when using this solution.
2. It is critical that the antibody is specific to the transgene because it is not possible to distinguish nonspecific interactions from specific ones in this method. The antibody used should react only with the transgene product in a western blot of transgenic kernels. If nonspecific interactions are detected, they can sometimes be removed by preadsorbing the antibody with nontransgenic endosperm as described in the optional method in **Subheading 3.2**.
3. A convenient way to make the tissue prints is to cut the modeling clay between each seed and pick each seed up by the clay. The goal is for the seed to be wet when it is blotted, but not so wet that the solution spreads on the blotting membrane. Different seeds soak up solution at different rates and the volume of seed extraction buffer and time to printing can be adjusted to account for this. If a seed does not leave a moist spot on the paper it is probably too dry to give a good signal. It can be remoistened with seed extraction buffer and blotted again.
4. The concentration of antibody should be optimized. Generally, a concentration that works well for a western blot will work well for this protocol. This procedure works with mono- or polyclonal antibodies.

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